

MYCOTOXINS

Rapid Immunochemical Screening Method for Aflatoxin B₁ in Human and Animal Urine

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A method has been developed to determine the presence of aflatoxin B₁ in the urine of animals (including humans) by utilizing commercial immunochemical kits that can be used in the field. Urine is treated with diatomaceous earth and filtered to clarify the sample; 2–3 ppb aflatoxin B₁, corresponding to about 300 ppb in the ingested feed/food, can be detected in the filtered urine without further purification. To improve sensitivity, the urine filtrate is passed through a C₁₈ solid phase column to extract the aflatoxin. The column is washed with acetonitrile-water (15 + 85) and water, aflatoxin B₁ is eluted with methanol-water (7 + 3), and water is added to the eluate, which is then tested for aflatoxin with the test kit. The limit of detection is 0.2 ppb, reflecting consumption of 40 ppb or more aflatoxin in the feed/food. When the initial sample volume is adequate, purification through the C₁₈ column step is usually sufficient. For limited sample volumes, the eluate from the C₁₈ column is mixed with water, added to an immunosorbent affinity column, and washed with water to remove excess sample matrix and impurities. Aflatoxin B₁ is eluted with acetonitrile. The extract is evaporated under nitrogen and the residue is redissolved in methanol-water (25 + 75). At this purification stage, the limit of detection is reduced to 0.05 ppb.

The aflatoxins are highly toxic and carcinogenic compounds found in food supplies grown in many areas of the world (1, 2). Aflatoxins have been found in mold-damaged foods, such as cereal grains, nuts and nut products, and edible oilseed products, and in milk and other dairy products. Although aflatoxin contamination is greatest in Asian and African countries, it is an annual problem to some degree in areas of the U.S. southeast, southern Midwest, and southwest. On occasion (e.g., 1983 and 1988), heavy contamination occurs in the midwestern corn-growing states. Animal feeding studies (beef, swine, poultry) have shown that edible meat becomes contaminated with aflatoxin when animals are fed contaminated corn (3–9).

Numerous methods that can be used to screen and/or quantitatively determine aflatoxins in many food matrices have been developed, collaboratively tested, and adopted by AOAC (10); however, no fast screening method is available for detecting aflatoxin in animal tissues. Federal guidelines permit feeding animals aflatoxin-contaminated grain at levels of less than 300 ppb (11). Therefore, producers and regulatory agencies have an imperative need for a screening method that can quickly and accurately identify contaminated edible meat to keep it from reaching the marketplace. An

accurate, sensitive, quantitative method for determining aflatoxin in animal tissue is available (12); however, it cannot be conveniently adapted to a qualitative method, nor is it a practical method for use as a screening procedure because it requires considerable time to complete.

An initial project to develop a screening method for the detection of aflatoxin residues in animal tissues that could be used by the Food Safety and Inspection Service (FSIS)/USDA was unsuccessful. An alternative approach involving development of a method to detect aflatoxin in animal urine was evaluated. Such a method would permit sample analysis that should have a high degree of correlation with tissue contamination (based on feeding studies) and that could be performed without sacrificing the animal. This paper describes a method for detecting aflatoxin in the urine of humans and other animals that permits the selection of any of 3 different minimum detection limits: 2–3, 0.2, or 0.05 ppb aflatoxin B₁.

METHOD

Equipment

(a) *Solid phase extraction (SPE) columns*.—Bond Elut C₁₈ cartridge, 6 mL (Cat. No. 607306, Analytichem International, Harbor City, CA, or equivalent).

(b) *SPE column adapter*.—Bond Elut adapter to join syringes to SPE columns (Cat. No. 636001, Analytichem International, or equivalent).

(c) *Affinity column (AF column)*.—Aflatest P (VICAM, Somerville, MA, or equivalent).

(d) *Filter paper*.—S&S No. 588, fast flow, high wet strength, 12.5 cm, or equivalent.

(e) *Vacuum manifold*.—Supelco vacuum manifold, or equivalent.

(f) *Aflatoxin test kit*.—EZ-Screen test kit, 3.3 ppb sensitivity (Environmental Diagnostics, Inc., Burlington, NC) or Afla-20 Cup Test Kit (International Diagnostics Inc., St. Joseph, MI). Follow instructions included in each kit. Briefly, sample extracts are added to the card containing a glass fiber filter with aflatoxin antibody immobilized on it. An enzyme conjugate (horseradish peroxidase-aflatoxin B₁) is added, followed by a water wash. A substrate (color reagent) is added and the card is read for the presence (no color) or absence (color) of aflatoxin B₁.

(g) *Syringes*.—1, 10, and 30 mL disposable Luer-Lok syringes.

(h) *Pipetter*.—50 μ L automatic pipet (Gilson, or equivalent).

Reagents

(a) *Solvents*.—Reagent grade acetonitrile and methanol.

(b) *Diatomaceous earth*.—Hyflo Super-Cel.

(c) *Aflatoxin B₁ and M₁ standards*.—Standard solutions containing 0.5 μ g/mL aflatoxin B₁ or M₁ in acetonitrile were

Received June 8, 1990. Accepted September 14, 1990.

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This paper was presented at the 102nd AOAC Annual International Meeting, August 29–September 1, 1988, at West Palm Beach, FL.

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prepared from concentrated solutions of crystalline aflatoxins.

(d) *Artificially contaminated urine.*—Standard aflatoxin B₁ solution was added by syringe to aflatoxin-free urine to obtain samples containing desired toxin levels.

Sample Treatment

Measure 60 mL bovine, porcine, or human urine into 125 mL glass-stoppered Erlenmeyer flask and add 4 g diatomaceous earth. Shake vigorously 30 s, filter through paper into a graduated cylinder, and collect 40 mL. Save for SPE extraction step or test for aflatoxin B₁ with test kit. Minimum detection limit is 2–3 ppb.

SPE Extraction

(Note: The SPE extraction and the affinity column chromatography steps may be performed without a vacuum manifold by applying pressure with a syringe and plunger. *Never* pull the plunger up without disconnecting the syringe from the column. Drawing solvents or air upward may ruin the test results.)

Equip C₁₈ cartridge with adapter and attach 30 mL syringe as sample reservoir. Attach cartridge to vacuum manifold and adjust vacuum to ca 10 in. Hg. Pretreat cartridge with 5 mL methanol followed by 5 mL H₂O. Do not allow cartridge to run completely dry hereafter unless so specified. Add 40 mL clarified urine sample from graduated cylinder to syringe barrel and draw solution through cartridge at flow rate of ca 20 mL/min. Rinse sample graduate with 3 mL H₂O and pass rinse through cartridge. Rinse cartridge with 5 mL acetonitrile-H₂O (15 + 85), ca 1 drop/s, and discard eluate. Wash cartridge with 5 mL H₂O (fast drops), and allow cartridge to run dry. Remove cartridge from manifold. If aflatoxin B₁ is to be assayed after elution from SPE cartridge, elute aflatoxin into 1 dram vial with 1.5 mL MeOH-H₂O (7 + 3), using syringe. Add 2 mL H₂O to eluate and measure with test kit. Minimum detection limit is 0.2 ppb. If urine extract from SPE cartridge is to be further purified by affinity column chromatography, elute aflatoxins from SPE cartridge with 2 mL MeOH-H₂O (7 + 3), and mix with 12 mL H₂O.

Affinity Column Chromatography

Remove cap from column top and clip off end to fit Luer tip of 10 mL syringe. Replace cap on column. Remove bottom cap and attach column to vacuum manifold. Add SPE cartridge extract (after mixing with 12 mL H₂O) and pull solution through affinity column (10 mL/min). Rinse sample container with 2 mL H₂O and pass rinse through column. Pull column dry for ca 5 s to remove excess H₂O. Elute aflatoxins from affinity column with 1 mL acetonitrile (use 1 or 2 mL syringe) into 1 dram vial, discarding first 3 drops. Evaporate eluate to dryness under N₂ at 40°C. Redissolve residue in 200 µL (0.2 mL) MeOH-H₂O (1 + 3), cap vial,

and mix vigorously for ca 1 min, preferably on a Vortex mixer. Save for aflatoxin test kit. Minimum detection limit is 0.05 ppb.

Results and Discussion

The results of the screening tests for the presence of aflatoxin B₁ in human, bovine, and porcine urine are given in Table 1. These data were obtained with the EZ Screen Card test manufactured by Environmental Diagnostics Inc., Burlington, NC. Other kits that should perform the screening tests satisfactorily are available, e.g., Afla-20 cup test (International Diagnostics Inc., St. Joseph, MI) and the CITE Probe test (IDEXX Corp., Portland, ME). Aflatoxin B₁ at the 1.5 ppb level in most raw clarified urine samples gave positive test results. Two bovine and 2 porcine urine samples tested negative at this concentration.

In a study of populations in the People's Republic of China, where a high incidence of liver cancer has been reported (2), between 1.23 and 2.18% of dietary aflatoxin B₁ was found to be excreted as aflatoxin M₁ in human urine. This corresponds to a similar ratio of M₁ found in milk of dairy cows fed aflatoxin B₁ (3). Less than 0.5% of the total B₁ intake was excreted as B₁; therefore, a factor of about 200 is indicative of the transmission differential between the aflatoxin B₁ intake in food/feed and is excretion in urine. The relationship between the B₁ intake and M₁ excretion in animals (6, 7) is similar to the human values published by Zhu et al. (2); therefore, it is likely that a similar B₁ excretion relationship also exists. Based on these transmission data, a mammal would need to consume a food source contaminated with 300 ppb aflatoxin B₁ before the toxin could be detected in raw, clarified urine. This may be satisfactory for some animals, since the FDA will allow aflatoxin levels of 300 ppb in corn to be fed to feedlot cattle (11).

Since a higher percentage of B₁ intake is excreted as M₁ than as B₁, a test kit for aflatoxin M₁ would be useful in this application because sensitivity would be increased 2- to 4-fold. However, at the time of this work, no rapid test kits were available for M₁. We evaluated the 2 test kits used in this study for cross activity to aflatoxin M₁ and found none with 40 ng of pure standard. When M₁ kits become available, it is highly probable that they can be substituted for the B₁ kits. This would significantly increase the sensitivity of the method.

An improvement in sensitivity can be obtained with a rapid cleanup step. After the various clarified urine specimens were passed through the SPE C₁₈ column and the adsorbed aflatoxin eluted from the column, the extracts produced positive tests at the 0.2 ppb level for nearly all urine specimens. The negative results shown in Table 1 (0.1 ppb bovine and porcine; 0.2 ppb porcine) occurred early in our experiments. Personal communications with Environmental Diagnostics International (EDI) revealed that the test kits are designed to give uniformly negative results at the level of 1.65 ppb B₁ and

Table 1. Results of screening artificially contaminated urine of humans and other animals for aflatoxin^a

Urine sample type	Positive aflatoxin B ₁ tests (No. positive/No. total tests)							
	Clarified raw urine		SPE C ₁₈ column extract			Affinity column extract		
	1.25 ppb	1.50 ppb	0.05 ppb	0.10 ppb	0.20 ppb	0.010 ppb	0.020 ppb	0.050 ppb
Human	1/8	8/8	0/12	6/6	—	1/6	6/6	6/6
Bovine	0/10	8/10	0/10	7/10	20/20	5/15	15/15	15/15
Porcine	—	11/13	0/10	7/10	19/20	6/12	10/13	15/15

^a As determined with EZ Screen Card Text (Environmental Diagnostics Inc., Burlington, NC).

uniformly positive results at the level of 3.3 ppb. All test lots meet this criterion or "window," but some lots will have a crossover point (change from negative to positive) nearer 3.3 ppb, while other lots will have a crossover nearer 1.65 ppb.

After we obtained fresh test kits, all results were positive. Obviously, the negative results mentioned above were obtained with kits that had a crossover point near 3.3 ppb, while the crossover point of new kits was nearer to 1.65 ppb. EDI indicated that their experiments have shown that there are no matrix effects with the kits; therefore, this window will exist with urine samples as well. In a test with the ability to detect 0.2 ppb aflatoxin B₁ in mammalian urine, an ingested food sample containing 40 ppb aflatoxin would produce a positive test for aflatoxin in urine. This presents a method with a satisfactory sensitivity, since the FDA guideline for aflatoxin in animal feeds ranges from 20 to 300 ppb.

If the volume of the urine sample is limited or if a lower detection limit is desired, an affinity column cleanup step may be added to the sample preparation. The data (Table 1) indicate that a sensitivity of 0.05 ppb aflatoxin in the urine of all 3 species can be achieved by the addition of this purification step. If the urine volume specified in the procedure (40 mL) is used, a concentration of 10 ppb aflatoxin B₁ in feed would cause a positive urine test with the immunochemical kits; however, a sensitivity of that magnitude is unnecessary. If only 4 mL of urine was available for analysis, however, this method would still detect B₁ in urine from an animal consuming food containing 100 ppb or more of aflatoxin B₁.

Several important and useful objectives are achieved with this method. First, it can be performed in the field; a syringe and plunger can be used to carry out the various column cleanup steps, and no expensive or complicated equipment is needed to complete the immunochemical tests. Second, the collection of the urine sample is noninvasive; a test specimen can be obtained without the need to puncture or surgically invade the body. Third, the test procedure is relatively inexpensive. No special equipment is needed and very little time is required to make the determination. For most tests, less than

30 min will be needed to clarify the urine, clean it up with a SPE C₁₈ column, and run the test to determine if aflatoxin is present. Finally, this method could easily be used by regulatory agencies to determine if animals had eaten aflatoxin-contaminated feed.

Acknowledgments

The authors thank H. M. Stahr, Iowa State University, Ames, IA; T. Keetin, Texas A & M University, College Station, TX; and J. Richard, formerly of National Animal Disease Center, Ames, IA, now at NRRRC, Peoria, IL, for supplying swine and beef urine.

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Supplied by U.S. Department of Agriculture, National Center for Agricultural Utilization Research, Peoria, IL